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Upregulation of junctional adhesion molecule-A is a putative prognostic marker of hypertension

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Aims Establishing biochemical markers of pre-hypertension and early hypertension could help earlier diagnostics and therapeutic intervention. We assess dynamics of junctional adhesion molecule-A (JAM-A) expression in rat models of hypertension and test whether JAM-A expression could be driven by angiotensin (ANG) II and whether JAM-A contributes to the progression of hypertension. We also compare JAM-A expression in normo- and hypertensive humans.

Methods and results In pre-hypertensive and spontaneously hypertensive rats (SHRs), JAM-A protein was overexpressed in the brainstem microvasculature, lung, liver, kidney, spleen, and heart. JAM-A upregulation at early and late stages was even greater in the stroke-prone SHR. However, JAM-A was not upregulated in leucocytes and platelets of SHRs. In Goldblatt 2K-1C hypertensive rats, JAM-A expression was augmented before any increase in blood pressure, and similarly JAM-A upregulation preceded hypertension caused by peripheral and central ANG II infusions. In SHRs, ANG II type 1 (AT_1) receptor antagonism reduced JAM-A expression, but the vasodilator hydralazine did not. Body-wide downregulation of JAM-A with Vivo-morpholinos in juvenile SHRs delayed the progression of hypertension. In the human saphenous vein, JAM-A mRNA was elevated in hypertensive patients with untreated hypertension compared with normotensive patients but reduced in patients treated with renin–angiotensin system antagonists.

Conclusion Body-wide upregulation of JAM-A in genetic and induced models of hypertension in the rat precedes the stable elevation of arterial pressure. JAM-A upregulation may be triggered by AT_1 receptor-mediated signalling. An association of JAM-A with hypertension and sensitivity to blockers of ANG II signalling were also evident in humans. We suggest a prognostic and possibly a pathogenic role of JAM-A in arterial hypertension.

Keywords Adhesion molecules • JAM-A • Hypertension • Angiotensin • Vasculature

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1. Introduction

In 2003, 'pre-hypertension' was officially recognized as a transitional step to the fully developed disease,[1] but what happens during this initial period is not clear. Moreover, the diagnosis of pre-hypertension rests exclusively on the detection of blood pressure over a certain range at rest (systolic pressure >120 and <140 mmHg), rather than on a biochemical marker. Such a marker could provide an earlier, more reliable, and accurate prognostic indicator for essential hypertension. Subtle pathological changes taking place throughout the body in pre-hypertension may form harmful positive feedback loops. For example, activated lymphocytes could cause vascular inflammation resulting in further activation of the immune system, tissue damage, and eventually in hypertension.[2–4] Junctional adhesion molecule-A (JAM-A), otherwise known as the F11 receptor or JAM-1,[5–7] belongs to the immunoglobulin superfamily. A transmembrane variant of JAM-A is expressed by endothelial cells, leucocytes, and platelets, but an isoform lacking the transmembrane domain also exists as a soluble protein in plasma.[8] In the endothelium and epithelium, JAM-A localized to cell–cell contacts is known to be involved in leucocyte/platelet/endothelial cell interactions.[9,10] We previously found an increased level of JAM-A mRNA in the brainstem nucleus tractus solitarii (NTS—a key autonomic control centre) of the spontaneously hypertensive rat (SHR)[11] in adult but also in young pre-hypertensive SHRs, indicating that it was unlikely to be a reaction to the pressure elevation. Local adenoviral overexpression of JAM-A in the NTS of normotensive rats resulted in a transient increase in blood pressure.[11] Since then, polymorphisms in JAM-A gene have been associated with human hypertension in a Hong Kong Chinese population,[12] suggesting that observations in SHRs are likely to be relevant to human hypertension. An elevated level of the soluble form of JAM-A was found in hypertensive patients.[13]

We hypothesized that the upregulation of JAM-A might be a biochemical marker of developing hypertension. A number of outstanding questions need to be addressed to support this notion. First, from previous studies,[11] it was not known to what extent changes in JAM-A mRNA manifest themselves at the protein level. It was unknown whether JAM-A upregulation observed in the SHR is specific only to this model or whether it is also characteristic of other animal models of hypertension (i.e. non-genetically pre-programmed) and human patients. We also wished to determine whether JAM-A upregulation is evident outside of the central nervous system and to what extent. Factors triggering JAM-A upregulation remained unknown and its possible involvement in the pathogenesis of hypertension required further investigation.

The objectives of the present study were three-fold. First, we have assessed JAM-A expression at the protein and mRNA level in a range of commonly used rat models of hypertension. JAM-A expression was also studied in tissues obtained from patients undergoing coronary artery bypass surgery (CABG) with normal and elevated blood pressure. Secondly, we tested the hypothesis that JAM-A upregulation could be a response to an increase in the activity of an angiotensin (ANG) II type 1 (AT_1) receptor signalling cascade, a characteristic feature of experimental and clinical hypertension. Finally, we tested whether a genetic antagonism of JAM-A in SHRs would have any effect on the onset of hypertension in this model.

2. Methods

An expanded 'Methods' section is available in the Supplementary material online.

2.1 Animal studies

Animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986, consistent with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011) and approved by the University of Bristol Ethical Review Group (UB/05/035). The numbers of animals used in individual experiments are provided in the Supplementary material online, *Table S1*, and are also indicated on the graphs.

Following the procedures, animals were anaesthetized using 4–5% isoflurane inhalation anaesthesia. Adequate anaesthesia was assured by the absence of reflexes prior to rapid cervical dislocation and tissues processed for real-time RT–PCR, immunohistochemistry, and western blot analyses of JAM-A (see Supplementary material online for details).

For the generation of the Goldblatt 2K-1C renovascular hypertension model, male Wistar rats were anaesthetized by intramuscular injection of a mixture of ketamine (75 mg/kg) and medetomidine (5 mg/kg). The adequacy of anaesthesia was determined by the loss of a pedal withdrawal reflex. The left renal artery was then partially obstructed with a silver clip, followed by implantation of a radio-transmitter into the abdominal aorta for telemetric measurement of arterial blood pressure. The renal arteries of control rats were not clipped but were exposed.

To establish ANG II-induced hypertension, 7 days after the implantation of a radio-transmitter into the abdominal aorta, male Wistar rats were implanted with an ANG II-containing Alzet osmotic pumps subcutaneously, which delivered at a rate of 200 or 50 ng/kg/min. In a separate group, Wistar rats were subcutaneously implanted with osmotic pumps, for intracisternal delivery (5 ng/kg/min). All control rats were infused with 0.9% saline.

To assess the role of ANG II in JAM-A upregulation, 12- to 15-week-old male SHRs were orally administered losartan at 30 mg/kg/day or hydralazine at 50 mg/kg/day for 4 weeks in drinking water. The doses were calculated and adjusted based on the rate of water consumption which was monitored and therefore the cited doses are approximations. The control rats were given drinking water. The tail arterial systolic blood pressure was determined with tail-cuff sphygmomanometer (IITC Life Science Inc.) before (week 0) and after 4 weeks of losartan or hydralazine administration.

2.2 Experiments in the rat brain endothelial cell line

The rat brain endothelial (RBE4) cell line was maintained with α-MEM/Ham's F-10 nutrient mixture (1:1). They were treated with ANG II, followed by real-time RT–PCR analysis of JAM-A mRNA. In some experiments, RBE4 cells were treated with an AT_1 receptor blocker losartan (10^{-5} M). Control cells were treated with vehicle only.

2.3 JAM-A expression in the human saphenous vein

Anonymized surplus small fragments of the human saphenous veins were obtained on the completion of CABG surgery carried out at the Bristol Heart Institute, from normotensive patients ($n = 4$), hypertensive patients without medication ($n = 4$), and hypertensive patients ($n = 15$) prescribed with angiotensin-converting enzyme (ACE)-inhibitor and/or ANG II receptor blocker. Protocols were approved by the local Ethics Committee and the consent of patients was obtained. JAM-A expression in the samples of the veins was assessed using real-time RT–PCR.

2.4 Fluorescence-activated cell sorting analysis of JAM-A expression on leucocytes and platelets

Blood was collected from the hearts of three euthanized adult, male Wistar rats and three SHRs. Staining performed with Alexa Fluor 700

(AF700)-conjugated mouse anti-rat CD4 (AbD Serotec), Phycoerythrin (PE)-conjugated mouse anti-rat CD54 (ICAM-1) (BD Pharmingen), and goat anti-rat JAM-A (Santa Cruz) antibodies. Erythrocytes were lysed using Pharm Lyse (BD Biosciences). The secondary CyTM5-conjugated Donkey anti-Goat IgG (Jackson ImmnuoResearch) antibody was used for JAM-A staining. All samples were tested in duplicate. The leucocyte population was identified by its scatter properties (the parent population). Platelets were isolated by centrifugation after dilution of lysed blood 1:1 by HEPES-Tyrode buffer to create a platelet-rich plasma layer which was used for fluorescence-activated cell sorting (FACS). Five thousand to 10 000 gated events were recorded for each sample. The geometric mean (average fluorescence of the population) and the percentage of parent population for the positive and negative groups were calculated using FlowJo V7.6 (TreeStar Inc.).

2.5 Downregulation of JAM-A with morpholino in SHRs

Morpholinos are synthetic oligonucleotide-like molecules which induce gene knockdown by forming stable complexes with the target mRNA. We have used a specially modified 'Vivo-morpholinos' which contain a unique delivery element. Vivo-morpholino targeting JAM-A molecule (5′-GTGCCCATTACAGTTTCAATCCCGA-3′) was complementary to the 5′ region of JAM-A (accession no: BC065309), including the start codon. As a control, we used Vivo-morpholino oligo (5′-CCTCT TACCTCAGTTACAATTTATA-3′) which has no known rodent RNA targets. Both oligos were purchased from Gene Tools LLC (Philomath, OR, USA) and maintained in sterile saline according to the manufacturer's instructions. Four-week-old SHRs (63 ± 2 g) were used. Systolic blood pressure was measured using tail cuff (see Supplementary material online for details) and anti-JAM-A or control morpholinos were administered via the tail vein. Two additional injections were made 5 and 10 days later (three injections in total, total dose 20 mg/kg), while systolic blood pressure was monitored weekly until 7 weeks of age. After that, SHRs were implanted with radiotelemetry transmitters and their level of blood pressure was assessed ~10 days later. Tissues were extracted and processed for western blotting to demonstrate JAM-A knockdown.

2.6 Data analysis

The data were assessed by analysis of variance (ANOVA) and/or a *t*-test where applicable using SPSS software package. Differences between groups were considered significant at $P < 0.05$. The data are presented as mean ± SEM.

3. Results

3.1 Upregulation of JAM-A protein in genetically pre-programmed hypertension in SHRs and stroke-prone SHRs

Previously we reported[11] the upregulation of JAM-A mRNA in the NTS of SHRs. Here, we confirmed that the JAM-A mRNA level is increased in the NTS of both young and mature SHRs and that in stroke-prone SHRs (SHRSP), which has a more severe hypertensive phenotype,[14] the JAM-A mRNA level was significantly higher than the SHRs (*Figure 1A*).

Immunofluorescence analysis revealed that JAM-A protein was increased in NTS, rostral ventro-lateral medulla (RVLM), and hypothalamic paraventricular nucleus (PVN) of SHRs compared with the Wistar-Kyoto Rat (WKY) and that levels in SHRSP exceeded those in the SHR (*Figure 1B* and *C*). For example, in NTS, JAM-A immunofluorescence intensity in mature WKY was 148 ± 9 U/section ($n = 6$), while in mature SHRs, it was 433 ± 29 U/section ($n = 6$, $P < 0.05$ vs. WKY, ANOVA). In SHRSP, levels increased further to 720 ± 45 U/section ($n = 4$, $P < 0.01$ vs. WKY, $P < 0.05$ vs. SHRs, ANOVA). Interestingly, in pre-hypertensive 3-week-old SHRs, JAM-A levels in NTS, RVLM, and PVN were much higher than in age-matched WKY rats (*Figure 1B* and *C*). JAM-A protein was upregulated throughout the brain including areas not usually involved in autonomic control, such as the hippocampus and the eighth cerebellar lobule (see Supplementary material online, *Figure S1*). Moreover, western blotting showed JAM-A upregulation in isolated brainstem blood vessels, lung, liver, kidney, spleen, and heart of SHRs, SHRSP, and young SHRs (*Figure 1D*; see Supplementary material online, *Figure S2*).

Since JAM-A is also expressed on leucocytes and platelets, we tested whether this upregulation can also be detected in these cells. FACS has revealed no differences between blood of mature WKY and SHRs in terms of the numbers of optically detectable leucocytes (data not shown). SHRs and WKY had a similar frequency of JAM cvc-A-positive leucocytes within the CD4+ population ($P = 0.64$), while there was a slight reduction in the frequency of JAM-A-positive CD54+ cells (from 29.8 ± 4.5 to 19.9 ± 1.4, $P = 0.02$; *Figure 1E*) in the SHR. Consistently, the level of fluorescence (estimated from the geometric mean) in SHR CD54+ cells was also significantly reduced (2453.5 ± 194.0 to 1490 ± 102.3 U, $P = 0.01$). There was no significant difference in JAM-A expression on platelets (data not shown).

3.2 Upregulation of JAM-A in renovascular hypertension

In a non-genetic Goldblatt 2K-1C-induced hypertension, a significant elevation in 24 h mean arterial pressure (MAP) was observed after 2 weeks of clipping and then rose further to plateau at week 5 (*Figure 2A*) as we reported previously.[15] After 1 week, when MAP had not yet elevated, JAM-A was upregulated in the brain including NTS, RVLM, and PVN (*Figure 2B*; see Supplementary material online, *Figure S3A–C*). The JAM-A level peaked at week 3 and did not increase further, despite the continued elevation in MAP (*Figure 2A* and *B*; see Supplementary material online, *Figure S3A–C*). Upregulation of JAM-A mRNA in NTS was detected as early as week 1 (*Figure 2C*), which was consistent with western blot data showing the elevation of JAM-A in all tested tissues (*Figure 2D*; see Supplementary material online, *Figure S4*).

3.3 Upregulation of JAM-A in hypertension induced by subcutaneous ANG II infusion

At a rate of 50 ng/kg/min, ANG II sc did not increase 24 h MAP in Wistar rats until the 10th day of infusion (*Figure 3A*). Nevertheless, this dose upregulated JAM-A protein in the brain microvasculature (NTS, RVLM, and PVN; see Supplementary material online, *Figure S5A*, *B*, *D* and *E*) by day 5. The level of JAM-A increased further by day 10 by which time the MAP became significantly different from the saline-treated group (*Figure 3A* and *B*; see Supplementary material online, *Figures S5A* through *D* and *S6*). Early upregulation of JAM-A at the mRNA level was also confirmed in the NTS (by 1.60 ± 0.07-fold at day 5 and by 2.00 ± 0.09-fold at day 10 of ANG II infusion; *Figure 3B*). Western blot revealed body-wide upregulation of JAM-A (see Supplementary material online, *Figures S5C* and *S6*).

ANG II infused at a higher rate (200 ng/kg/min sc) significantly increased MAP by day 4 and pressure continued rising until day 10

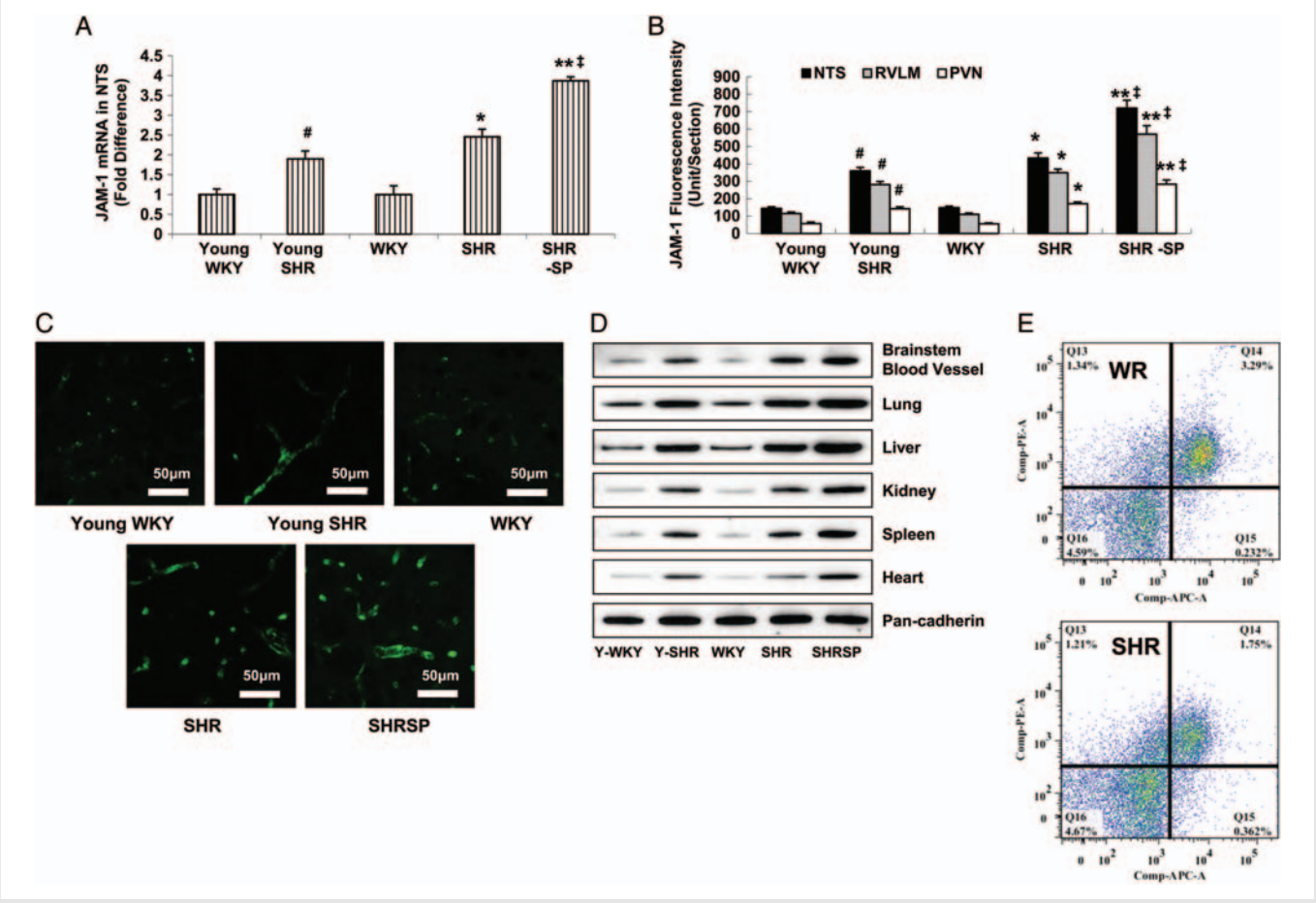

Figure 1 Overexpressed JAM-A is associated with genetically pre-programmed hypertension in the SHR and SHRSP. (*A*) JAM-A mRNA in NTS of young SHRs (Y-SHR, pre-hypertensive), SHRs (adult and hypertensive), SHRSP, and age-matched normotensive WKY rats. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. WKY rats. $^{\#}P < 0.05$ vs. young WKY(Y-WKY) rats. $^{\ddagger}P < 0.05$ vs. SHRs. (*B*) Immunofluorescence intensity analysis of JAM-A expression in NTS, RVLM, and PVN from same rat groups as in (*A*). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. WKY rats. $^{\#}P < 0.05$ vs. young WKY rats. $^{\ddagger}P < 0.05$ vs. SHRs. (*C*) Representative confocal images of JAM-A in NTS from same rat groups as in (*A*). (*D*) Western blots of JAM-A protein (~35 kDa band) in isolated tissues from same rat groups as in (*A*) with pan-cadherin as a membrane protein loading control. (*E*) Representative FACS charts from a WKY and SHRs to illustrate a slight reduction in CD54+/JAM-A-positive population of leucocytes in SHRs.

(*Figure 3C*). In these animals, JAM-A protein was dramatically upregulated by day 5 but in contrast to MAP, no further significant increase occurred after day 5 (see Supplementary material online, *Figures S5F*, *S7A–D*, and *S8*). A similar pattern was evident at the mRNA level (*Figure 3D*).

3.4 Central infusion of ANG II upregulates JAM-A

Intracerebroventricular infusion of ANG II at 5 ng/kg/min did not significantly increase MAP of Wistar rats until day 10 (*Figure 3E*). Nevertheless, after 5 days of infusion, JAM-A mRNA in the NTS was elevated by 1.37 ± 0.07-fold ($n = 4$, $P < 0.05$ vs. saline control, ANOVA) and it further increased by 1.80 ± 0.09-fold by day 10 ($n = 4$, $P < 0.01$ vs. control, $P < 0.05$ vs. day 5, ANOVA; *Figure 3F*). This pattern of JAM-A upregulation was also evident in RVLM and PVN (see Supplementary material online, *Figure S9A* and *B*). Western blotting confirmed body-wide JAM-A upregulation already at days 5 and 10 of ANG II icv infusion (see Supplementary material online, *Figure S5G*).

3.5 JAM-A expression in SHRs treated with losartan or hydralazine

We tested how a reduction in pressure in SHRs may impact on the level of JAM-A. In SHRs, tail arterial systolic pressure was significantly reduced ($n = 8$, $P < 0.01$, ANOVA) by oral administration of losartan (30 mg/kg/day) for 4 weeks (*Figure 4A*). This was accompanied by a downregulation of JAM-A mRNA in RVLM, PVN, and NTS (see Supplementary material online, *Figure S10A–C*) and a body-wide reduction in the high JAM-A protein characteristic of the SHR (*Figure 4B*). To differentiate between the effect of blocking AT_1 receptors vs. lowering arterial pressure, hydralazine was infused (50 mg/kg/day for 4 weeks). It significantly decreased systolic pressure in SHRs (*Figure 4C*) but did not change JAM-A protein in any of the tested tissues (*Figure 4D*) or JAM-A mRNA levels in RVLM, PVN, and NTS (see Supplementary material online, *Figure S10D–F*).

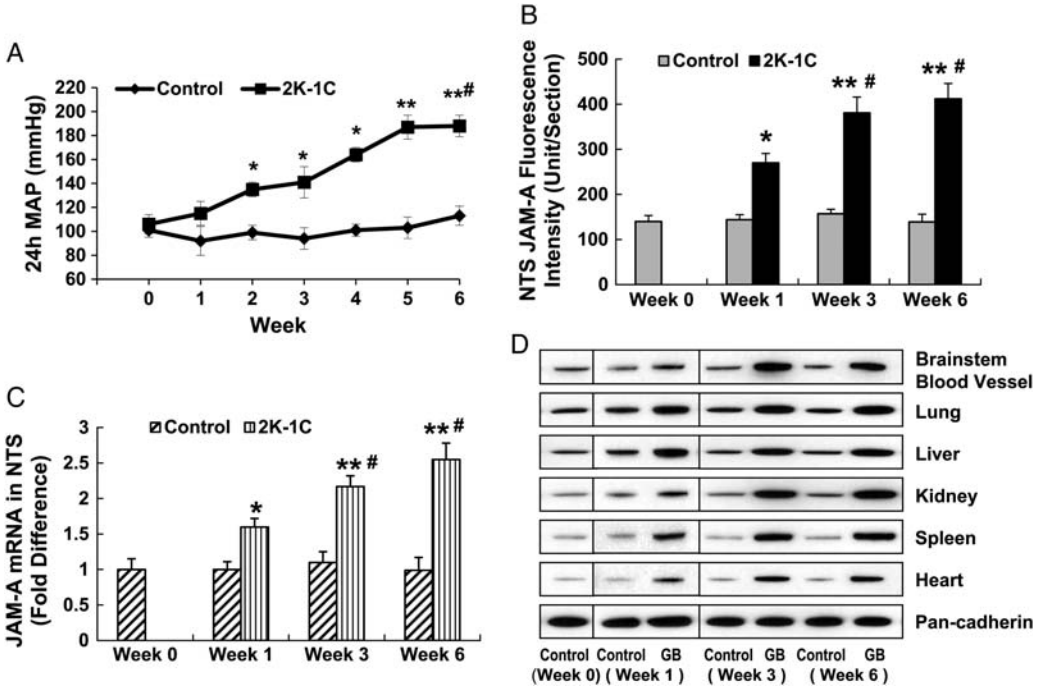

Figure 2 JAM-A is overexpressed in a Goldblatt (GB) 2K-IC renovascular hypertensive rat model. (*A*) Time course of 24 h MAP of Wistar rats after clipping and sham-operated control rats ($n = 12$ per time point). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control. $^{\#}P < 0.05$ vs. week 3. (*B*) Immunofluorescence intensity analysis of the JAM-A level in NTS from Goldblatt 2K-1C and control rats. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control. $^{\#}P < 0.05$ vs. week 1. Group 'week 0' are untreated rats from the same batch. Four animals were processed for immunofluorescence, real-time PCR, and western blotting from each group at 1-, 3-, and 6-week post-renal artery clipping. (*C*) JAM-A mRNA in NTS of Wistar rats of the Goldblatt 2K-1C model and control rats. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control. $^{\#}P < 0.05$ vs. week 1. (*D*) Western blots of JAM-A protein in isolated tissues of Goldblatt 2K-1C control rats. Lanes 2 and 3 and 4–6 were run on the same gels.

3.6 JAM-A is upregulated in hypertensive human patients

In veins of patients without a history of hypertension but diagnosed as hypertensive upon admission (blood pressure >150/100 mmHg, $n = 4$), JAM-A mRNA was upregulated by 4.86 ± 0.55-fold (*Figure 4E*) compared with normotensive patients (blood pressure <140/90 mmHg, $n = 4$). In veins from hypertensive patients treated with either an ACE-inhibitor or an ANG II receptor blocker or their combinations with other anti-hypertensive agents ($n = 15$), the JAM-A mRNA level was higher than in normotensive subjects but lower than in untreated patients (*Figure 4E*; see Supplementary material online, *Table S2*).

3.7 ANG II upregulates JAM-A *in vitro*

We hypothesized that elevated renin–angiotensin system (RAS) activity could be one of the factors responsible for JAM-A upregulation. This was investigated *in vitro* using the RBE4 rat brain endothelial cell line. JAM-A mRNA in RBE4 cells was elevated by ANG II in a concentration-dependent manner (10^{-8}–10^{-6} M) for 24 h (*Figure 5A*). Increased expression of JAM-A became apparent after 8 h of ANG II treatment (*Figure 5B*). Pre-treatment with losartan (10^{-5} M) almost completely prevented the stimulatory effect of ANG II (*Figure 5C*).

3.8 Morpholino-induced JAM-A knockdown delays the progression of hypertension in the SHR

The early upregulation of JAM-A in all the rat models of hypertension tested here suggested that it could contribute to the establishment of the pathological level of blood pressure. To attenuate the expression of JAM-A in the SHR, we used a specially formulated '*vivo*' morpholino which was administered to young SHRs during the critical period between 4 and 8 weeks of age when hypertension develops. The progression of hypertension in SHRs treated with morpholinos was consistently and significantly attenuated as evidenced using both tail-cuff and radio-telemetry. In contrast, in animals treated with the control morpholino, blood pressure increased to the levels characteristic of an untreated SHR (*Figure 6*; see Supplementary material online, *Figure S11*). The effect of JAM-A targeting morpholino was confirmed by western blotting showing reduced JAM-A protein (see Supplementary material online, *Figure S12*). Finally, we tested the level of blood pressure in JAM-A knockout mice[16] but found that it was comparable to wild-type littermates, which is probably due to compensation (data not shown).

4. Discussion

This study was based on the previous evidence for JAM-A mRNA upregulation in the brainstem of SHRs.[11] Here, we have confirmed

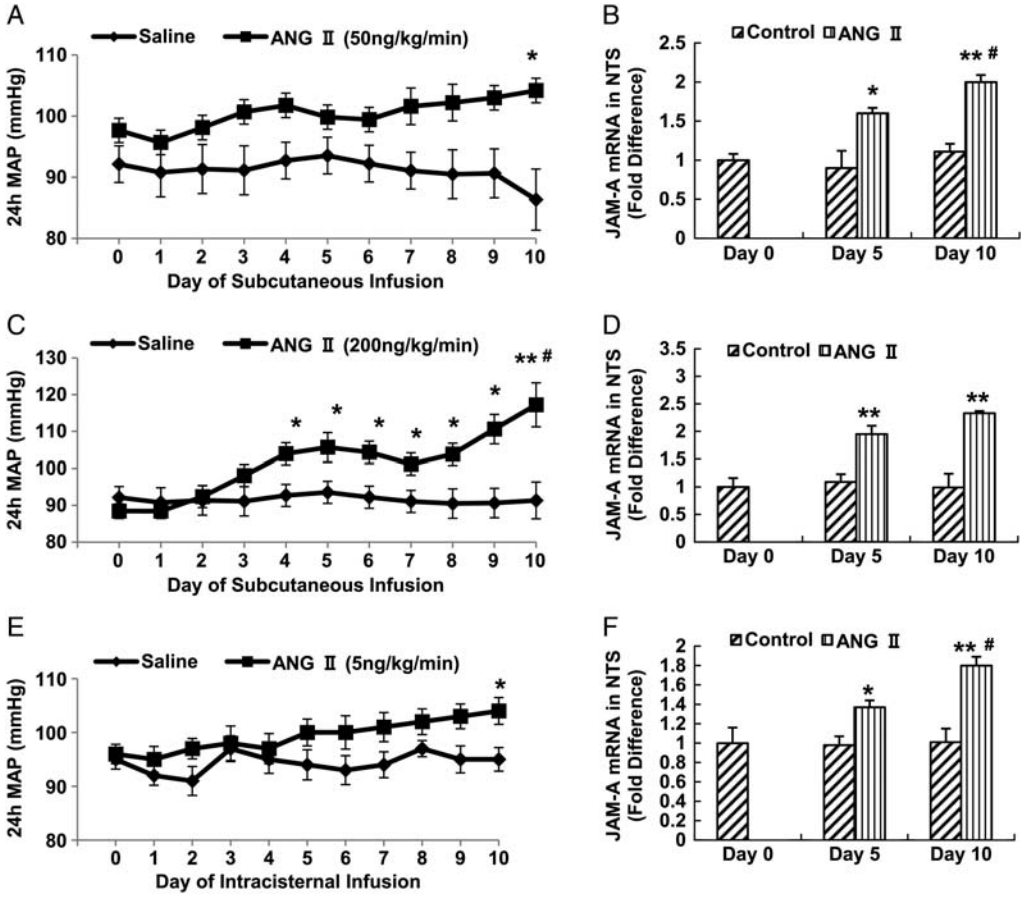

Figure 3 JAM-A expression in Wistar rats is elevated ubiquitously after subcutaneous and intracisternal infusion of ANG II infusion of ANG II. (*A*) Time course of 24 h MAP in Wistar rats subcutaneously infused with vehicle (control) and ANG II at 50 ng/kg/min. $n = 12$, $^{*}P < 0.05$ vs. saline control ($n = 12$). (*B*) JAM-A mRNA in NTS of Wistar rats subcutaneously infused with ANG II at 50 ng/kg/min. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. saline control. $^{\#}P < 0.05$ vs. day 5. (*C*) Time course of 24 h MAP of Wistar rats subcutaneously infused with vehicle (control) or ANG II at 200 ng/kg/min. $n = 12$, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. saline control. $^{\#}P < 0.05$ vs. day 5. (*D*) JAM-A mRNA in NTS of Wistar rats subcutaneously infused with ANG II at 200 ng/kg/min. $^{**}P < 0.01$ vs. saline control. (*E*) Time course of 24 h MAP of Wistar rats infused intracisternally with ANG II at a rate of 5 ng/kg/min. $n = 8$, $^{*}P < 0.05$ vs. saline control. (*F*) JAM-A mRNA in NTS of Wistar rats infused intracisternally with vehicle (control) or ANG II at 5 ng/kg/min. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. saline control. $^{\#}P < 0.05$ vs. day 5.

these observations at the mRNA level, extended them to the protein level, and found that JAM-A upregulation in this genetic model of hypertension is not selective for any particular part of the brain and is detectable throughout the whole body. This includes organs clearly implicated in blood pressure/volume regulation such as the kidneys but also others, such as the liver. NTS, PVN, and RVLM were specifically picked up for the JAM-A level measurement as they play major roles in the neuro-hormonal regulation of blood pressure. Hence, the expression of JAM-A protein seems to be increased in all endothelial cells in the SHR and, consistent with our previous data, is already evident in young, pre-hypertensive animals, indicating that it is not secondary to the hypertension. Mature SHRs had the same relative level of JAM-A protein as young SHRs, while the strongly hypertensive phenotype of SHRSP was associated with the greatest levels of JAM-A supporting an association between this protein and the severity of hypertension. We acknowledge that in tissues such as kidneys or lung part of JAM-A protein could include a contribution from epithelial cells, which are known to also express this protein[17,18]; however, brain microvessels were specifically purified and we did not notice any clear staining outside of blood vessels, indicating that these measurements only reflect changes in the endothelium. In addition, the non-vascular myocardium is also devoid of JAM-A (Professor E. Dejana, Institute of Molecular Oncology, Milan, personal communication). Finally, the ability of losartan to regulate JAM-A expression via AT1 receptors requires that the target cells express this receptor. To the best of our knowledge, AT_1 receptors are not described in the epithelium of the lung or other organs studied here. Therefore, changes in JAM-A expression in various tissues reported here may be expected to reflect expression in the endothelium rather than epithelium.

We have also found JAM-A upregulation at mRNA and protein levels in two non-genetic models of hypertension: the 2K-1C and ANG II infusion (peripheral and central). Consistent with the data from the pre-hypertensive SHR, JAM-A protein upregulation in both of these models cannot be secondary to hypertension because JAM-A levels always increased prior to a significant elevation of MAP. Moreover, in 2K-1C, once high levels of JAM-A protein were established at around 3 weeks, it did not significantly rise further

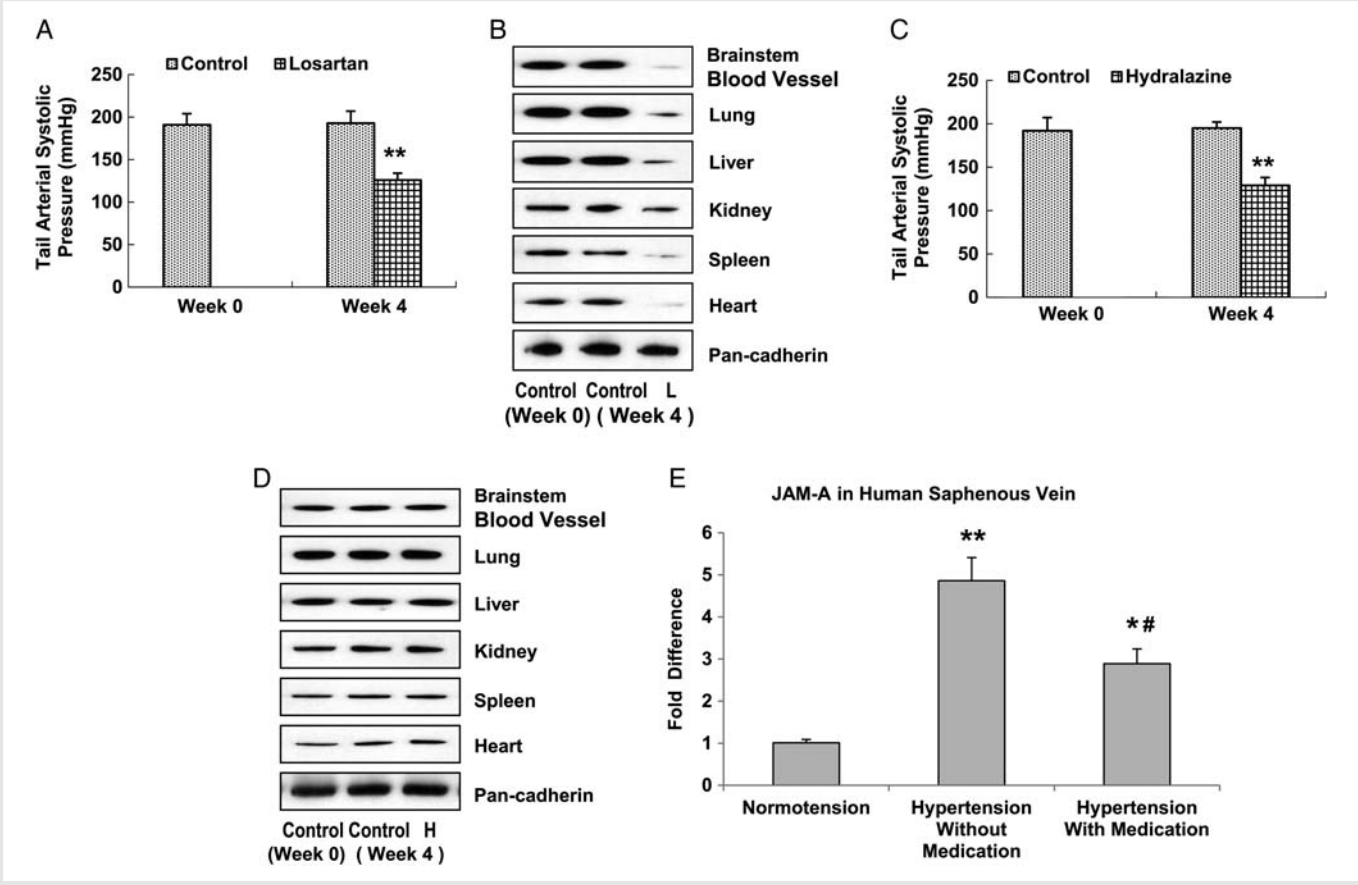

Figure 4 Effect of losartan and hydralazine on JAM-A expression in SHRs (*A*–*D*) and JAM-A expression in the human saphenous vein from hypertensive patients (*E*). (*A*) Tail arterial systolic blood pressure of SHRs orally administered losartan or drinking water. $n = 8$, $^{**}P < 0.01$ vs. vehicle control. (*B*) Western blots of JAM-A protein in isolated tissues of SHRs. (*C*) Tail arterial systolic blood pressure of SHRs orally administered hydralazine or drinking water. $n = 8$, $^{**}P < 0.01$ vs. vehicle control. (*D*) Western blots of JAM-A in isolated tissues of SHRs. $n = 4$ per group. (*E*) JAM-A mRNA in saphenous veins of normotensive patients ($n = 4$), hypertensive patients without medication ($n = 4$), and hypertensive patients treated with ACE-inhibitors and/or AT_1 receptor blockers ($n = 15$). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. normotension. $^{\#}P < 0.05$ vs. hypertension without medication. See Supplementary material online, *Table S2*, for further information on the treatments.

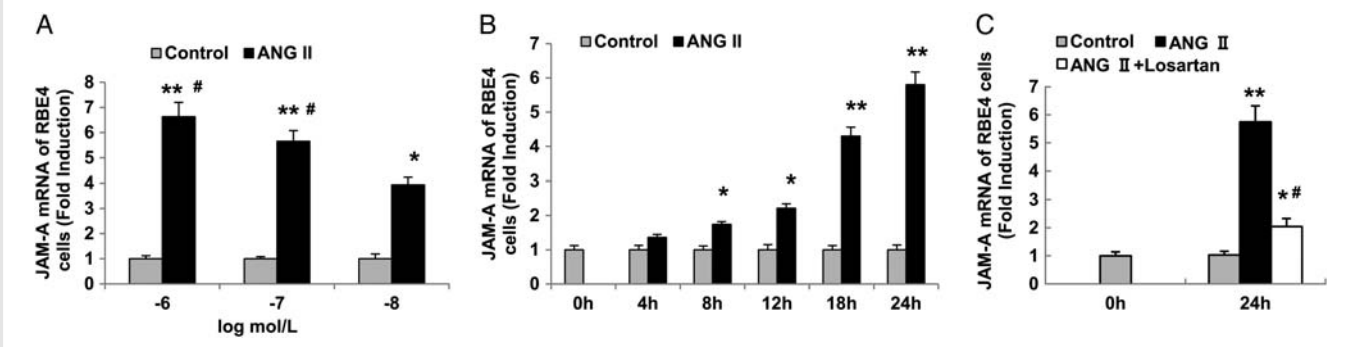

Figure 5 ANG II drives JAM-A expression in RBE4 rat brain endothelial cells. (*A*) JAM-A mRNA in RBE4 cells increases after doses of ANG II from 10^{-6} to 10^{-8} M; no changes were seen following the treatment with vehicle. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control. $^{\#}P < 0.05$ vs. 10^{-8} M. (*B*) JAM-A mRNA in RBE4 cells treated with ANG II at 10^{-7} M for various durations (h). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. vehicle control. (*C*) JAM-A mRNA in RBE4 cells pre-treated with losartan at 10^{-5} M for 1 h, followed by stimulation with ANG II at 10^{-7} M for 24 h. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. vehicle control. $^{\#}P < 0.05$ vs. ANG II. Results are mean ± SEM of four duplicates in each group.

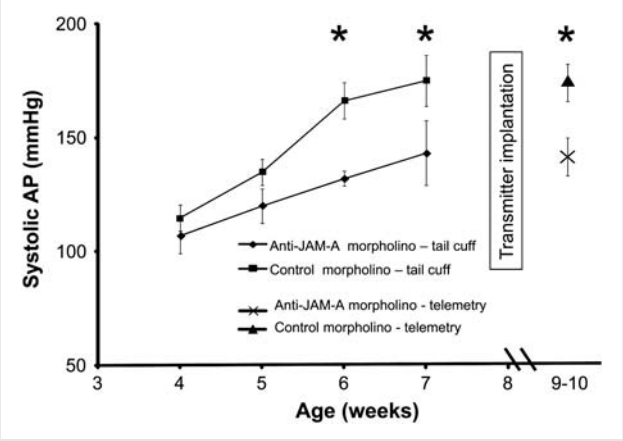

Figure 6 Effect of anti-JAM-A morpholino on arterial blood pressure in young SHRs. Systolic blood pressure (BP) was assessed using tail-cuff during weeks 4–7. After that, animals were implanted with radio-transmitters and their BP was assessed using telemetry 1 week later (weeks 9–10). Diastolic BP and mean BP were also decreased according to telemetry (see Supplementary material online, *Figure S12*). $*P < 0.05$ (ANOVA), $n = 4–6$ rats per data point.

despite a dramatic increase in arterial pressure between 3 and 6 weeks post-clipping. Similarly, in rats infused with 50 ng/kg/min, ANG II JAM-A expression increased by day 5, yet at the arterial pressure only became marginally different from control by day 10.

Interestingly, even hypertension induced by central infusion of ANG II is preceded by increased expression of JAM-A, and this is detectable in both central and peripheral blood vessels. It has been recently shown that central ANG II might activate T-lymphocytes triggering a generalized immune response which possibly acts in a feed-forward manner to further rise blood pressure.[2] JAM-A upregulation could therefore facilitate this mechanism as it is involved in leucocyte adhesion and transmigration.[19] It may also increase platelet adhesion to the endothelium potentially leading to inflammatory thrombosis.[20] Such effects of JAM-A could facilitate the development of hypertension, and consistent with this idea, downregulation of JAM-A in young SHRs with morpholino delayed the usual age-dependent progression of hypertension. We acknowledge that this knockdown was not targeted to the endothelium and was body-wide. Nevertheless, the present results are consistent with the pro-hypertensive role of a high level of JAM-A as was originally hypothesized.[11] The possible pathogenic role of JAM-A-related intracellular signalling in hypertension requires further investigation.

Several observations from this study suggest that ANG II might be one of the factors responsible for JAM-A upregulation in hypertension. First, JAM-A is upregulated by ANG II *in vitro* in the RBE4 cell line. The effect of ANG II on JAM-A level is concentration-dependent and can be blocked by losartan. Secondly, when the SHR is treated with losartan, JAM-A expression is reduced in parallel with the fall in arterial pressure. This was not due to hypotension because hydralazine, which decreased blood pressure similarly to losartan, did not affect JAM-A levels. Thirdly, the hypertensive patients in the cohort treated with ACE-inhibitors and/or AT_1 receptor blockers had lower expression of JAM-A in the saphenous vein compared with those diagnosed hypertension but not treated with these drugs.

If it is the local tissue RAS which is responsible for the upregulation of JAM-A, there should not necessarily be a correlation between circulating levels of renin or ANG II with expression of JAM-A. The present study demonstrated that central infusion of ANG II leads to an increase in JAM-A levels throughout the body. This suggests that centrally induced hypertension can trigger the activation of local RAS in the peripheral tissues, or alternatively, JAM-A upregulates in response to an increase in sympathetic activity triggered by central ANG II. There is a consensus that ANG II may trigger vascular inflammation by inducing oxidative stress and upregulation of pro-inflammatory transcription factors such as nuclear factor kappa B (NF-κB). These in turn regulate expression of inflammatory mediators (e.g. C-reactive protein, chemokines, and adhesion molecules) which leads to endothelial dysfunction and vascular injury characteristic of sustained hypertension.[21–23] Intriguingly, a molecule with somewhat overlapping functions, the intercellular adhesion molecule-1 (ICAM-1), can be upregulated by ANG II in the brain endothelium *in vitro* via an AT_1 receptor/NF-κB pathway.[24] The structure of the JAM-A gene is quite complex, but its promoter also contains a consensus for NF-κB, providing a converging pathway for ANG II modulation.[25] Moreover, endothelial ICAM, JAM-A, and platelet endothelial cell adhesion molecule were demonstrated to act together to facilitate neutrophil transmigration through the activated endothelium[26] and it is possible that they represent a cascade pathologically activated in pre-hypertension and the early stages of hypertension. At these stages, the upregulation of JAM-A could facilitate vascular inflammation resulting from T-lymphocyte activation[2,3] and help to form the vicious positive feedback circle leading to hypertension. Interestingly, no upregulation of JAM-A was detected in leucocytes or platelets by FACS analysis, suggesting that the phenomenon is driven by a specific signalling cascade. Since leucocytes and platelets do not express AT1 receptors, it could be the reason why these cell types are not a part of the generalized JAM-A upregulation, which is driven by ANG II as our data suggest.

In summary, the present study demonstrates that body-wide upregulation of JAM-A at mRNA and protein levels occurs in several widely used rat models of hypertension 'before' the arterial pressure deviates from control. JAM-A also contributes to the establishment of hypertension in SHRs. Importantly, there is a big increase in JAM-A mRNA in venous tissue from hypertensive human patients which can be partially off-set by anti-hypertensive medication involving blockade of the RAS. The upregulation of JAM-A prior to the onset of the sustained elevation of MAP suggests that it reflects genetic or biochemical reorganization which occurs in the pre-hypertensive state, likely under the influence of subpressor levels of ANG II.

5. Clinical perspective

This study demonstrates that early body-wide upregulation of JAM-A is a unifying feature of several commonly used rat models of hypertension. Moreover, a high level of JAM-A is sustained when the hypertension fully develops. Strong upregulation of the endothelial form of JAM-A in human patients shown here is consistent with the recent reports of both an increase in concentration of the soluble isoform of JAM-A in human hypertension and association of the polymorphisms in JAM-A gene with obesity and hypertension in man. Because JAM-A upregulation precedes sustained elevation of blood pressure, these observations raise an intriguing possibility of using JAM-A as an early biochemical marker of the pre-hypertensive state. Moreover,

since real-time PCR requires only minute quantities of tissue, there might be a possibility to establish a clinically applicable test for JAM-A expression in biopsy samples from peripheral tissues. Further studies are needed to establish whether upregulation of the soluble variant of JAM-A also is detectable in pre-hypertension.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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